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SHORT COMMUNICATION

Arthropod moulting hormones (ecdysteroids) are present in the blood of insectivorous bats

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ABSTRACT

Ecdysteroids are steroid hormones involved in moulting and development of arthropods. Blood samples of 32 individual bats of eight insectivorous species were analysed for the presence of ecdysteroids, using liquid chromatography and mass spectrometry. Nine ecdysteroids were detected. The spectrum of these ecdysteroids was similar in bat species which take their preferred food items from the same insect order. The spectrum of blood-borne ecdysteroids was broader in the autumn than in the summer, and higher concentrations of 20-hydroxyecdysone (the most common ecdysteroid) occurred in samples from large bat species than from small ones. Ecdysteroids may have anabolic effects on insectivorous bats and may also affect their blood-feeding arthropod parasites.

INTRODUCTION

Bats (Chiroptera) have attracted enormous scientific attention on account of their biological–ecological properties and epidemiological significance. Their ability to fly has entailed unique anatomical and physiological mechanisms, including high metabolic rates (Munshi-South & Wilkinson 2010), short digestive tracts (Caviedes-Vidal et al. 2007), and even modifications in their immune functions (Brook & Dobson 2015). Owing to their flying activity and feeding habits, bats play indispensable roles in temperate and tropical ecosystems, as pollinators among nectar-feeding bats (Fleming et al. 2009) and regulators of insect populations among insectivorous bats (Charbonnier et al. 2014). Bats often represent the highest individual numbers and most diverse group of wild mammals living in urban habitats (Jung & Threlfall 2016), and their epidemiological role has long been subject to investigations.

Insects, on which the great majority of bat species feed, contain arthropod moulting hormones, so-called ecdysteroids

(Riddiford & Truman 1993). Low to high concentrations of (mostly) insect-derived ecdysteroids occur in the blood of insectivorous passerine birds (Hornok et al. 2019). Because passerine birds and bats share adaptive mechanisms in connection with flying (e.g. high metabolic rate and short digestive tract), and insectivory is also among their common traits, we hypothesised that ecdysteroids may also be present in the blood of insectivorous bats. The aim of this study was to investigate this possibility.

METHODS

Origin of samples

Blood samples were taken from 32 bats representing eight insectivorous species from two families (Appendix S1; Fig. 1). The first group of blood samples (n = 14) were collected from bats in Bulgaria and Romania, in early September 2017, and at the end of July and the beginning of August 2018. Samples came from a further 18 bats (Appendix S1), which were transported to the Animal Rescue Centre of Budapest Zoo between mid-August 2017 and early September 2018. Weight characteristics and feeding preferences of bat species are included in Appendices S2 and S3, respectively.

Target ecdysteroids and analysis methods

Blood samples were tested for the presence of ten natural ecdysteroids. The standards of these compounds were obtained from previous phytochemical studies (Hunyadi et al. 2007, 2016). Ultra-high-performance liquid chromatography and high-resolution quadrupole-orbitrap mass spectrometry methods were used. Measurements were performed on an Acquity I-Class UPLC System (Waters, Milford, MA, USA) coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

The ultra-high-performance liquid chromatography separation was carried out on an Accucore C30 column (150 × 2.1 mm, 2.6 µm) with an equivalent guard column (10 × 2.1 mm, 2.6 µm; Thermo Fisher Scientific). The mobile phase A consisted of 0.1% formic acid aqueous solution, and mobile phase B was composed of acetonitrile with 0.1% v/v formic acid. The total run time was 29 min, and the following gradient programme was used: 0 min 10% B held for 1.5 min; ramped to 35% B in 18.5 min; then ramped to 100% B in 0.1 min; held for 4.9 min; and, finally, returned to initial conditions within 4 min. The flow rate was 0.4 ml/min during the analysis. The column temperature was maintained at 50°C, and the

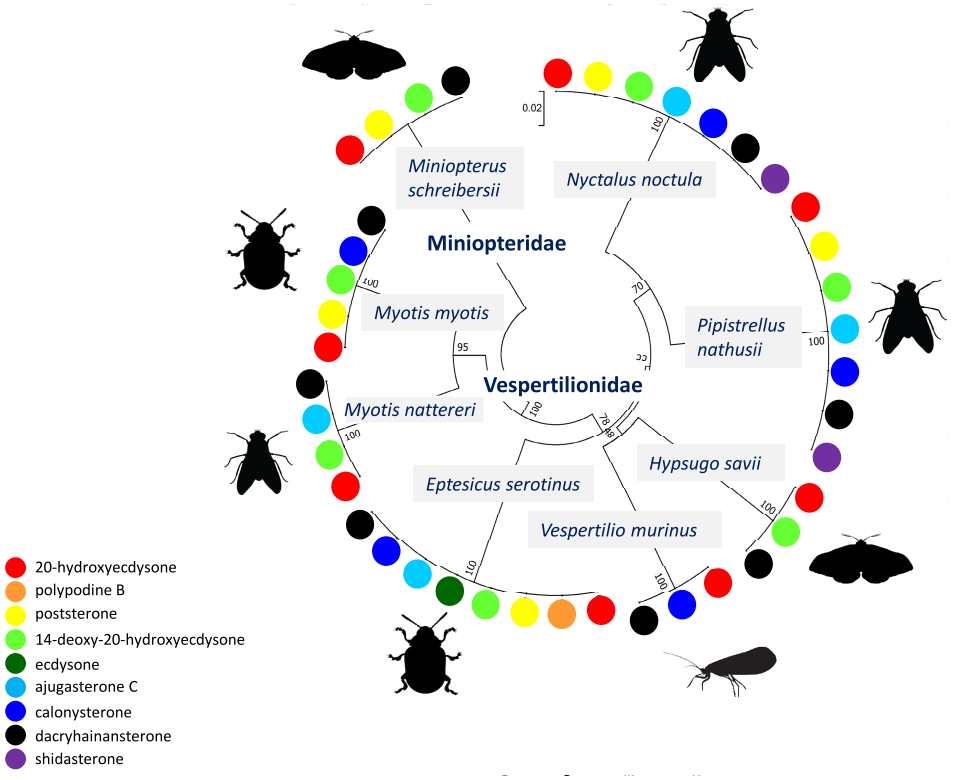

Fig. 1. Diversity of ecdysteroids found in the blood of eight bat species, and their predominant insect food items (beetles, order Coleoptera; moths, order Lepidoptera; flies and mosquitoes, order Diptera; and caddisflies, order Trichoptera). The topology is based on a cytochrome *b* phylogenetic tree.

injection volume was 8 l. Each sample was measured three times. For further details, see Appendices S4–S9.

RESULTS

Nine ecdysteroids were detected in the blood of eight insectivorous bat species (Fig. 1; Table 1). Ecdysteroids were the most diverse in *Eptesicus serotinus*, followed by *Nyctalus noctula* and *Pipistrellus nathusii* (Fig. 1). Considering the whole study period, the spectra of ecdysteroids were identical or overlapping in bat species which take their predominant food items from the same insect order (Appendix S3). In particular, all compounds detected in *Hypsugo savii* were also present in *Miniopterus schreibersii* (preferentially feeding on Lepidoptera); all compounds shown to be present in *Myotis myotis* were also demonstrated in *Eptesicus serotinus* (preferentially feeding on beetles); and *Myotis nattereri* shared all its compounds with *Nyctalus noctula* and *Pipistrellus nathusii* (Fig. 1). In the latter two species, the spectrum of ecdysteroids was the same, in accordance with their most preferred food items, Diptera, but despite the highly significant difference between their mean body weight (Appendix S2; 28.1 ± 3.63 g, n = 192 vs. 6.76 ± 1.48 g, n = 44, respectively; t = 62.015, degrees of freedom = 170.38, $P < 0.0001$). The mean number of compounds per sampling occasion was significantly higher during the autumn (median = 6, IQR = 2.5, n = 7 occasions; t = 2.34, degrees of freedom = 26, P = 0.028) than during the summer (median = 4, IQR = 1.25, n = 20 occasions; Appendix S1).

The predominant ecdysteroid (detected in all individual bats and reaching the highest concentrations) was 20-hydroxyecdysone (20E; Table 1). Among large bat species, the mean 20E concentration was significantly higher in *Eptesicus serotinus* (232.82 ± 287.23 pmol/ml, n = 8) than in *Nyctalus noctula* (38.49 ± 27.92 pmol/ml, n = 11; W = 77, P = 0.012). In addition, the mean concentration of 20E was significantly higher in bat species predominantly feeding on beetles (order Coleoptera; 204.96 ± 260.09 pmol/ml, n = 10) than in those typically feeding on flies (order Diptera; 49.74 ± 59.58 pmol/ml, n = 15; W = 129, P = 0.009; Fig. 1).

DISCUSSION

In this study, the presence of a broad range of naturally acquired ecdysteroids was demonstrated in the blood of eight insectivorous bat species, to the best of our knowledge for the first time worldwide. Hitherto, the general view persisted that experimentally administered ecdysteroids are rapidly cleared from the blood of mammals, e.g. mice *Mus musculus* (Dinan & Lafont 2006). However, the continuous uptake of insect food can apparently counterbalance this rapid clearance in bats.

The predominant source of ecdysteroids detected in bats is likely to have been insects, considering that all eight bat species investigated here are insectivorous. None of the species is known to feed on fruits, but they could still have indirect access to plant-derived ecdysteroids via their insect prey items that feed on plants. Several insect species are known to render dietary ecdysteroids inactive through conjugating them with long-chain fatty acids (Diehl et al. 1985). Such conjugates are non-toxic to the insect and may accumulate. Considering the abundance of non-specific esterases in mammals, it is a logical hypothesis that these esters act as prodrugs, releasing the free ecdysteroid in insectivorous animals such as bats. This food-chain-dependent transmission may explain the surprising occurrence of phytoecdysteroids (i.e. ecdysteroids that are not known to be naturally produced by insects, such as polypodine B, ajugasterone C, calonysterone, dacryhainansterone, and shidasterone; Dinan 2001), in bat blood as shown here.

Based on our results, identical spectra of detected ecdysteroids in *Nyctalus noctula* and *Pipistrellus nathusii* were in accordance with their common predominant food items, but not with their significantly different body weights. Assuming that the spectrum of blood-borne ecdysteroids is related to the taxonomic diversity of insect food items, these results confirm previous observations suggesting that dietary diversity is not related to the body mass of bats (Feldhamer et al. 2009). Variations in the spectra of ecdysteroids between sampling months might reflect changes in the daily or seasonal availability or uptake of insects from various taxonomic orders.

Based on the dataset of this study, within the category of large bats, higher 20E concentrations were measured in *Eptesicus serotinus* than in *Nyctalus noctula*. *Eptesicus serotinus* (unlike *Nyctalus noctula*) belongs to the category of 'ground-gleaning' bats (Catto et al. 1996); i.e., it obtains a significant portion of its food items from the surface of objects such as the ground and its plant covering. From such surfaces, *Eptesicus serotinus* predominantly feeds on beetles, which might contribute significantly to its high 20E blood concentrations. In beetles, the adult diapause is induced by the commencing short-day photoperiod in the autumn, concomitantly with ecdysteroid peak values nearly as high as those during metamorphosis (De Loof et al. 1984). In a broader context, this may also explain why the blood of bat species predominantly feeding on beetles contained significantly higher concentrations of 20E than that of those species that typically feed on flies.

In conclusion, this is the first demonstration of naturally acquired ecdysteroids in the blood of insectivorous bats, showing significant diversity and sometimes reaching high concentrations. Regarding the potential physiological implications of these findings, various ecdysteroids were experimentally shown to have a significant anabolic effect in

Table 1. Mean ecdysteroid concentrations in the blood of eight insectivorous bat species (pmol/ml; mean ± SD). Abbreviations of the 10 compounds' names are as follows: 20E – 20-hydroxyecdysone, pB – polypodine B, pS – poststerone, 14d – 14-deoxy-20-hydroxyecdysone, 2d – 2-deoxy-20-hydroxyecdysone, E – ecdysone, AjC – ajugasterone C, Cal – calonysterone, Dac – dacryhainansterone, and Shd – shidasterone. The number in parenthesis after ecdysteroid concentration refers to the sample size, when this is different from the number in the first column. The acronym 'ND' indicates that the relevant ecdysteroid was not detected

Bat species (sample size)	20E	pB	pS	14d	E	AjC	Cal	2d	Dac	Shd
Nyctalus noctula (11)	38.5 ± 27.9	ND	14.7 ± 6.1 (5)	8.7 ± 4.1 (6)	ND	9 ± 4 (3)	24.6 ± 32.1 (4)	ND	25.2 ± 18.6 (5)	7.1, 4.4
Pipistrellus nathusii (3)	96 ± 130.1	ND	273.4, 11.4	130.7, 6.6	ND	9.2	55.8	ND	9.5, 9.6	5.3
Hypsugo savii (1)	48.1	ND	ND	5.2	ND	ND	ND	ND	9.8	ND
Vespertilio murinus (2)	8.7, 12.1	ND	ND	ND	ND	ND	43.4	ND	5.3	ND
Eptesicus serotinus (8)	232.8 ± 287.2	22.2 ± 17.2 (4)	23.8 ± 22.5 (3)	5.9 ± 2.7 (6)	148.4 ± 104.6 (3)	10.5 ± 9.5 (3)	8.8 ± 3.9 (5)	ND	7.1 ± 5.5 (7)	ND
Myotis nattereri (1)	34.6	ND	ND	6.7	ND	4.2	ND	ND	10	ND
Myotis myotis (2)	104.8, 82.2	ND	12.5	7.1, 8.6	ND	ND	18.8	ND	6, 5.5	ND
Miniopterus schreibersii (4)	27.7 ± 22.1	ND	10.4, 19.8	5.2 ± 0.5 (3)	ND	ND	ND	ND	7 ± 5.3	ND

mammals (Dinan & Lafont 2006), sometimes exceeding that of doping agent steroids. For instance, 20E enhances physical performance in humans, acting as a 'natural dope' (Parr et al. 2014), and it is currently under consideration by the World Anti-Doping Agency as a doping-controlled substance. Therefore, the anabolic effect of these biologically active compounds in bats (if any) is worth further investigation. In addition, the oral uptake of excess amounts of ecdysteroids by blood-sucking ectoparasites is known to affect them in several ways (Rees 2004). Since insectivorous bats have significantly lower ectoparasite loads than sympatric fruit-eating bats (Luguterah & Lawer 2015), it is an important future line of research to examine whether blood-borne ecdysteroids influence the ectoparasite burdens of bats, as reported in insectivorous birds (Hornok et al. 2016).

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DATA AVAILABILITY STATEMENT

The raw data of this study are available at Mendeley Data, V1, https://doi.org/10.17632/jrr54sdr49.1.

REFERENCES

Brook CE, Dobson AP (2015) Bats as 'special' reservoirs for emerging zoonotic pathogens. *Trends in Microbiology* 23: 172–180.

Catto CMC, Hotson AM, Racey PA, Stephenson PJ (1996) Foraging behaviour and habitat use of the serotine bat (*Eptesicus serotinus*) in southern England. *Journal of Zoology* 238: 623–633.

Caviedes-Vidal E, McWhorter TJ, Lavin SR, Chediack JG, Tracy CR, Karasov WH (2007) The digestive adaptation of flying vertebrates: high intestinal paracellular absorption compensates for smaller guts. *Proceedings of the National Academy of Sciences of the United States of America* 104: 19132–19137.

Charbonnier Y, Barbaro L, Theillout A, Jactel H (2014) Numerical and functional responses of forest bats to a major insect pest in pine plantations. *PLoS One* 9: e109488.

De Loof A, Briers T, Huybrechts R (1984) Presence and function of ecdysteroids in adult insects. *Comparative Biochemistry & Physiology* 79B: 505–509.

Diehl PA, Connat JL, Girault JP, Lafont R (1985) A new class of apolar ecdysteroid conjugates: esters of 20-hydroxy-ecdysone with long-chain fatty acids in ticks. *International Journal of Invertebrate Reproduction and Development* 8: 1–13.

Dinan L (2001) Phytoecdysteroids: biological aspects. *Phytochemistry* 57: 325–339.

Dinan L, Lafont R (2006) Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. *Journal of Endocrinology* 191: 1–8.

Feldhamer GA, Carter TC, Whitaker JO Jr (2009) Prey consumed by eight species of insectivorous bats from southern Illinois. *American Midland Naturalist* 162: 43–51.

Fleming TH, Geiselman C, Kress WJ (2009) The evolution of bat pollination: a phylogenetic perspective. *Annals of Botany* 104: 1017–1043.

Hornok S, Csorba A, Kováts D, Csörgő T, Hunyadi A (2019) Ecdysteroids are present in the blood of wild passerine birds. *Scientific Reports* 9: 17002.

Hornok S, Kováts D, Flaisz B, Csörgő T, Könczöl Á, Balogh GT, Csorba A, Hunyadi A (2016) An unexpected advantage of insectivorism: insect moulting hormones ingested by song birds affect their ticks. *Scientific Reports* 6: 23390.

Hunyadi A, Gergely A, Simon A, Tóth G, Veress G, Báthori M (2007) Preparative-scale chromatography of ecdysteroids of *Serratula wolffli andrae*. *Journal of Chromatographic Science* 45: 76–86.

Hunyadi A, Herke I, Lengyel K, Báthori M, Kele Z, Simon A, Tóth G, Szendrei K (2016) Ecdysteroid-containing food supplements from *Cyanotis arachnoidea* on the European market: evidence for spinach product counterfeiting. *Scientific Reports* 6: 37322.

Jung K, Threlfall CG (2016) Urbanisation and Its effects on bats — a global meta-analysis. In: Voigt C, Kingston T (eds) *Bats in the Anthropocene: Conservation of Bats in a Changing World*, 13–33. Springer International Publishing, Cham, Switzerland.

Luguterah A, Lawer EA (2015) Effect of dietary guild (frugivory and insectivory) and other host characteristics on ectoparasites abundance (mite and nycteribiid) of chiropterans. *Folia Parasitologica* 62: 1–21.

Munshi-South J, Wilkinson GS (2010) Bats and birds: exceptional longevity despite high metabolic rates. *Ageing Research Reviews* 9: 12–19.

Parr MK, Zhao P, Haupt O, Ngueu ST, Hengevoss J, Fritzemeier KH et al. (2014) Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone. *Molecular Nutrition & Food Research* 58: 1861–1872.

Rees HH (2004) Hormonal control of tick development and reproduction. *Parasitology* 129(Suppl. 1): S127–S143.

Riddiford LM, Truman JW (1993) Hormone receptors and the regulation of insect metamorphosis. *American Zoologist* 33: 340–347.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

Appendix S1. Ecdysteroid concentrations according to bat species, sex, sample group and sampling month/year.
Appendix S2. Body weights (g) of bat species involved in this study, calculated from 2046 bats captured at various locations in Romania in the period 2015-2020.
Appendix S3. Phylogenetic and statistical analyses.
Appendix S4. Chemical structures of the ecdysteroids.
Appendix S5. Sample preparation and calibration.
Appendix S6. Conditions of operation of the mass spectrometer.
Appendix S7. The monitored m/z values of protonated compounds with related retention times.
Appendix S8. Ion chromatograms of ecdysteroids (extracted with the ultra-high-performance liquid chromatography and high-resolution quadrupole-orbitrap mass spectrometry method) in standard solution (A) and whole blood sample of a rescued *Pipistrellus nathusii* female, sampled in August (B).
Appendix S9. HRMS/MS fragmentation patterns of the investigated ecdysteroids.